Effects of sub-minimum inhibitory concentration antibiotic levels and temperature on growth kinetics and outer membrane protein expression in *Mannheimia haemolytica* and *Haemophilus somnus*

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Abstract

The objective of this study was to determine the effects of sub-minimum inhibitory concentrations (sub-MICs) of 2 veterinary antibiotic preparations, chlortetracycline (CTC) and chlortetracycline-sulfamethazine (CTC + SMZ), on growth kinetics and outer membrane protein expression in *Mannheimia haemolytica* and *Haemophilus somnus* at normal and febrile body temperatures. Sub-minimum inhibitory concentrations of both antibiotics reduced the growth rates of *M. haemolytica* and *H. somnus*. Growth of both species was not inhibited when grown at 41°C compared to 37°C. There was no detectable consistent effect of antibiotic or temperature on outer membrane protein expression for either species. Our study indicates that sub-MIC levels of CTC and CTC + SMZ markedly impair growth of clinical *M. haemolytica* and *H. somnus* isolates, potentially allowing more effective host clearance during infection.

Résumé

Cette étude avait pour but de déterminer les effets de concentrations inférieures aux concentrations minimales inhibitrices (sous-CMI) de deux préparations vétérinaires d'antibiotiques, la chlortétracycline (CTC) et la combinaison chlortétracycline-sulfaméthazine (CTC+SMZ), sur la cinétique de croissance et l'expression de protéines de la membrane externe de Mannheimia haemolytica *et* Haemophilus somnus *à deux températures de croissance. Les concentrations sous-CMI des deux antibiotiques ont réduit les taux de croissance de* M. haemolytica *et* H. somnus. *La croissance des deux espèces n'a pas été inhibée lors de la culture à 41 °C contrairement à ce qui s'est produit lors de la culture à 37 °C. La température de croissance et l'antibiotique n'ont eu aucun effet détectable sur l'expression des protéines de la membrane externe chez les deux espèces. Cette étude démontre que des niveaux sous-CMI de CTC et de CTC + SMZ affectent de façon marquée la croissance de d'isolats cliniques de* M. haemolytica *et* H. somnus, *permettant potentiellement une élimination plus efficace par l'hôte lors d'infection.*

(Traduit par Docteur Serge Messier)

Introduction

Stress that results from overcrowding and poor ventilation during shipping predisposes cattle to bacterial and viral respiratory infections. This stress-mediated disease complex is referred to as bovine respiratory disease (BRD), pneumonic pasteurellosis, or shipping fever. During BRD, bacteria infect the lower airways and induce an inflammatory reaction that is characterized by extensive infiltration of neutrophils and exudation of fibrin into pulmonary airways and alveoli (1). The result is extensive fibrinosuppurative and necrotizing bronchopneumonia (2).

Mannheimia haemolytica, *Pasteurella multocida*, and *Haemophilus somnus* are the bacteria most commonly associated with BRD. Of these, *M. haemolytica* biotype A serotype 1, a nonmotile, gram-negative, aerobic bacterium, is the most important etiologic agent of BRD (3). While *M. haemolytica* normally exists at low levels as a commensal in the nasopharynx of healthy calves, it is readily isolated from stressed cattle and cattle suffering from BRD (4). *Haemophilus somnus* is a gram-negative, pleomorphic species that is commensal in the bovine genitourinary tract and upper respiratory tract and acts as an opportunistic pathogen. In addition to its association with BRD, *H. somnus* causes a variety of bovine disease syndromes, including infertility, abortion, pneumonia, septicemia, arthritis, myocarditis, and thrombotic meningoencephalitis.

Antibiotic therapy has proven effective when initiated quickly after detection of clinical BRD. Moreover, prophylactic use of feed additive antibiotics significantly reduces morbidity and mortality rates due to BRD (5). However, the basis for the clinical benefit of prophylactic antibiotic use has not been determined.

Chlortetracycline (CTC), a broad-spectrum antibiotic, is commonly employed to treat BRD and to control BRD prophylactically. Chlortetracycline's mode of action involves binding to the 30S ribosomal subunit and inhibiting translation by blocking the attachment of aminoacyl transfer RNA to the mRNA-ribosome complex. Like other antibiotics that inhibit protein synthesis, CTC is bacteriostatic, and its ultimate effectiveness depends to some extent on active host

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resistance to the pathogen (6). Therefore, short-term impairment of bacterial reproduction by CTC should potentiate immunological elimination of the pathogen.

AUREO S 700 (Alpharma; Fort Lee, New Jersey, USA) is a proprietary antibiotic that is employed to aid in maintenance of weight gains in the presence of BRD. It is composed of a combination of CTC and sulfamethazine in a 1:1 ratio (CTC + SMZ). Sulfamethazine is a structural analog of para-aminobenzoic acid (PABA); it competitively inhibits the enzyme dihydropteroate synthetase, which catalyzes the binding of PABA to pteridine in the initial stage of folate synthesis. Therefore, the effectiveness of many sulfonamides is dependent on their ability to compete with PABA for dihydropteroate synthetase (6). The resulting decrease in folate concentration negatively affects bacterial growth by impairing folic acid-mediated synthesis of purines and pyrimidines.

The minimum inhibitory concentration (MIC) of an antibiotic is defined as the lowest concentration of the compound that completely inhibits the initiation of growth of a particular bacterium under standardized in vitro conditions. It has been reported that antibiotics at sub-MICs have numerous effects on bacteria, including morphological changes, modifications of cell wall structure, altered growth kinetics, inhibition of enzyme or toxin production, and loss of adhesive properties (7). Effects of sub-MICs of antibiotics on bacterial virulence factor expression may provide additional information for the rational use of antimicrobials in clinical practice (8).

The overall purpose of this study was to determine the effects of sub-MICs of CTC and CTC + SMZ on growth kinetics and outer membrane protein (OMP) expression in *M. haemolytica* and *H. somnus*. Selected strains (1 reference strain and 2 clinical isolates) of each species were analyzed with regard to in vitro sensitivities to CTC and CTC + SMZ at 37°C and 41°C. Subsequently, growth kinetics and OMP expression were determined at 1/2, 1/4, 1/8, and 1/16 MICs. Capsule expression was also measured at the same antibiotic levels for one of the *M. haemolytica* clinical isolates.

Materials and methods

Bacterial strains and growth conditions

Mannheimia haemolytica ATCC 55518 was included in the study as a nonvirulent control. Strain 55518 is an attenuated vaccine strain (*aroA* mutant) derived from a virulent pneumonic pasteurellosis isolate from cow lung (9). *Mannheimia haemolytica* clinical isolates D80 and D152 were originally isolated in 1983 from pneumonic bovine lungs at the Iowa State University Veterinary Diagnostic Laboratory. These isolates are sensitive to a broader range of antibiotics than current *M. haemolytica* isolates tend to be, but their virulence is comparable to current *M. haemolytica* clinical isolates (B. Briggs, personal communication). *Haemophilus somnus* ATCC 700025 is a bovine isolate used for antimicrobial sensitivity testing (10). *Haemophilus somnus* clinical isolates Hs-91 and 2336 were isolated from pneumonic bovine lung; Hs-91 was isolated from calf lung during an experimental challenge in 1991 at Iowa State University, and 2336 is a virulent isolate from pneumonic calf lung (11).

Mannheimia haemolytica strains were cultured on brain heart infusion (BHI) agar plates and batch cultures were cultivated in BHI broth. *Haemophilus somnus* was cultured on chocolate agar plates and batch cultures were cultivated in veterinary fastidious medium (VFM) (12). Agar plates were incubated for 18 h at 37°C (with 10% CO_2 for *H. somnus*) and stored at 4°C for no longer than 7 d. Stationary phase broth cultures were incubated at either 37°C (standard in vitro culture condition) or 41°C (simulating febrile body temperature) with rotary aeration at 180 rpm. Bacterial densities were estimated by measuring the optical density (OD) at 620 nm.

Antibiotic stock preparations

The CTC solutions (256 $\mu g\ mL^{-1}$) were prepared in BHI or VFM. The CTC + SMZ solutions (256 $\mu g\ mL^{-1}$ each) were prepared in the same manner as the CTC solutions with the addition of sulfamethazine in a 1:1 ratio, thereby simulating the commercial preparation AUREO S 700 (Alpharma). Media were filter sterilized and used the same day.

Minimum inhibitory concentration determinations

A serial 2-fold macro-broth dilution method was performed to determine the MICs of CTC and CTC + SMZ for *M. haemolytica* and *H. somnus* (12). Stationary-phase cultures of all strains were prepared at both 37°C and 41°C and used to inoculate fresh 5.0-mL cultures to an OD_{620} of 0.05. The 5.0-mL cultures were then incubated at either 37°C or 41°C until an OD_{620} of 0.10 was achieved, from which standardized bacterial suspensions were prepared to a final cell density of 5×10^5 cfu mL^{-1}.

Serial 2-fold dilutions were prepared from antibiotic stock solutions in BHI or VFM, and 1.0 mL of each standardized bacterial suspension was added to an equal volume of each antibiotic dilution (excluding the sterility control). After incubation for 24 h ± 1 h at either 37°C or 41°C, turbidity of the cultures was assessed visually by comparison to uninoculated controls. The MIC was defined as the lowest concentration of antibiotic where bacterial growth was not detected. The MICs were determined from independent triplicate assays and were based on a serial 2-fold plus or minus system (12). To be considered valid, MIC determinations for each of the 3 replicates had to be within plus or minus 1 dilution of each other; if necessary, additional replicates were run until 3 replicates were obtained within these limits.

Growth kinetics

The growth kinetics of all strains were determined in the presence of each sub-MIC at both 37°C and 41°C. Stationary-phase cultures were prepared at both 37°C and 41°C and used to inoculate 75 mL of BHI or VFM broth to an initial OD_{620} of 0.05. These cultures were divided into 5.0-mL aliquots and antibiotic solutions were added to yield desired sub-MICs. Cultures were incubated until they reached stationary phase, and bacterial densities were estimated turbidimetrically at 30 min intervals.

Growth kinetic assays for each *M. haemolytica* strain were performed in triplicate from the same stationary-phase starter culture and antibiotic stock solution. Triplicate growth curves were then duplicated using independent stationary-phase starter cultures and antibiotic stocks. Growth kinetic assays for each *H. somnus* strain

Table I. Minimum inhibitory concentrations (μg mL^{-1}) of chlortetracycline (CTC) and chlortetracycline-sulfamethazine (CTC + SMZ) for *Mannheimia haemolytica* and *Haemophilus somnus* strains at 37°C and 41°C

Bacterial strain	CTC 37°C	CTC 41°C	CTC + SMZ 37°C	CTC + SMZ 41°C
M. haemolytica				
55518	1	0.25	1	0.5
D80	16	16	32	16
D152	16	16	32	16
H. somnus				
700025	1	< 0.25	2	< 0.25
Hs-91	1	1	1	0.25
2336	4	4	4	2

were performed in triplicate from separate stationary-phase starter cultures and separate antibiotic stock solutions.

Generation times were estimated graphically from growth curves by plotting the average OD_{620} for each time point and manually drawing a straight line through the straightest portion of the growth curves and determining the time (in minutes) required for the OD_{620} to double. A minimum of 4 time points was used from each growth curve to determine the generation times.

Capsule staining technique

A slight modification of an acidic polysaccharide staining procedure (13) was employed to stain *M. haemolytica* strain D80 to determine the effects of sub-MIC antibiotic levels on capsule expression. Overnight cultures of *M. haemolytica* D80 were cultivated in the presence of sub-MICs of either CTC or CTC + SMZ, as described above. A 2.0 μL volume of each overnight bacterial cell culture was mixed with 2.0 μL of 1% aqueous Congo red on a microscope slide and allowed to air dry. Smears were counterstained without fixation for 2 min by flooding the slides with acid fuchsin. Each slide was then drained, blotted dry with filter paper, and examined by light microscopy. Multiple frame pictures were taken, and the images were digitized. The thickness of capsular material, for a minimum of 100 bacteria, was determined for each treatment by measuring the distance in micrometers from the outside edge of the capsular material to the outside edge of the bacterium (14) using computer software (Image-Pro; Media Cybernetics, Silver Spring, Maryland, USA). The data were analyzed using an analysis of variance (ANOVA) for a randomized complete block design with subsampling using a mixed procedure (SAS system for Windows, version 8; SAS, Cary, North Carolina, USA). The experimental date was the blocking factor and the frames within the microscope slide were the sub-samples. The treatment means were separated using the least significant differences. Confidence intervals were calculated to assess the biological importance of differences between the treatment means. The 5% level of significance was used for all tests.

Outer membrane protein extraction

Outer membrane proteins were isolated from 50-mL stationary-phase BHI cultures of the 3 *M. haemolytica* and the 3 *H. somnus* strains under each antibiotic and temperature combination in duplicate using a previously described protocol (15). Cultures containing appropriate antibiotic concentrations were incubated at 37°C or 41°C and pelleted by centrifugation at 12 000 × *g* for 20 min. Bacterial pellets were washed once in 25 mL of 10.0-mM HEPES buffer (pH 7.4), resuspended in 10 mL of 10-mM HEPES buffer, and sonicated on ice 4 times for 15 s each. The sonicated samples were then centrifuged at 1700 × *g* for 20 min at 4°C. The supernatant was retained and centrifuged at 100 000 × *g* for 60 min at 4°C. Following this centrifugation, the supernatant was discarded, and the pellet was resuspended in 1 mL of 10-mM HEPES buffer and 1 mL of 2% sodium N-lauroyl sarcocinate. The samples were incubated at room temperature for 30 min, followed by another centrifugation at 100 000 × *g* for 60 min at 4°C. The supernatant was discarded, and the pellet was resuspended in 400 μL distilled water. Protein concentrations were determined using the bicinchoninic acid (BCA) assay, by determining the OD_{562} in the OMP extracts and comparing that to known concentrations of bovine serum albumin using computer software (SoftMax Pro, version 2.1.1; Molecular Devices, Sunnyvale, California, USA).

Polyacrylamide gel electrophoresis (PAGE)

Samples consisting of 8 to 10 μg from each OMP preparation were resolved electrophoretically using sodium dodecyl sulfate (SDS)-PAGE with a 12% separating gel and a 4% stacking gel. Gels were stained with the Coomassie Brilliant Blue (Coomassie Brilliant Blue R-250 Staining Solutions Kit; Bio-Rad Laboratories, Hercules, California, USA). The SDS-PAGE was performed for each of the duplicate OMP preps from each *M. haemolytica* and *H. somnus* strain at each antibiotic and temperature combination. The approximate molecular weights of major and minor bands were determined by plotting the log molecular weights of the protein standards versus the distance each band migrated and then performing a linear regression on each of the standard curves.

Results

Minimum inhibitory concentrations

The MICs of both CTC and CTC + SMZ for all strains examined at 37°C and 41°C are shown in Table I. The MICs for both CTC and CTC + SMZ for *M. haemolytica* 55518 were at least 16 times lower than those for the 2 clinical isolates, regardless of temperature. Strain 55518 was 4-fold more susceptible to CTC at 41°C compared to 37°C, and all 3 strains were 2-fold more susceptible to CTC + SMZ at 41°C

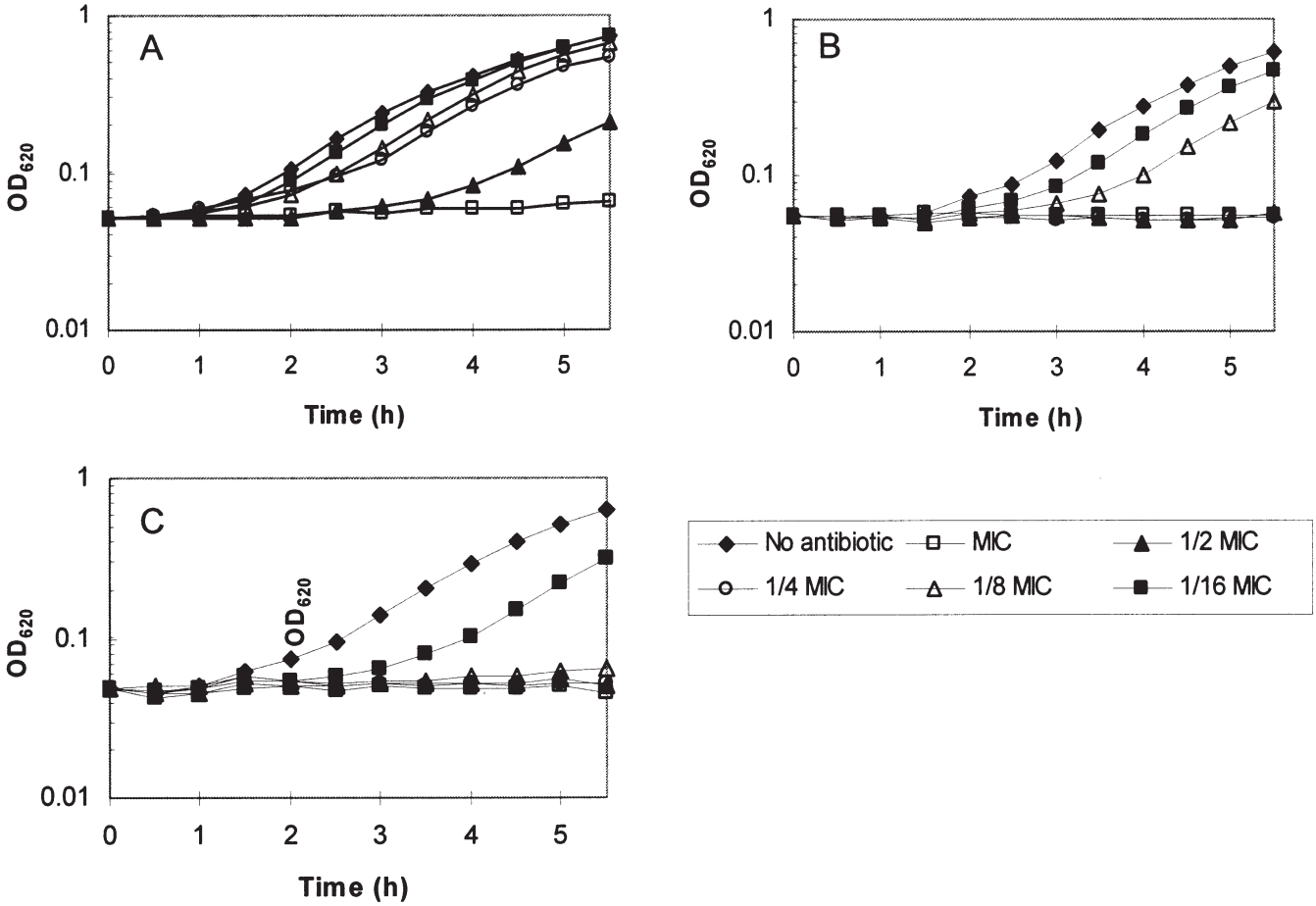

Figure 1. Growth kinetics of *Mannheimia haemolytica* strains 55518 (A), D80 (B), and D152 (C) in the presence of sub-minimum inhibitory concentrations (MICs) of chlortetracycline (CTC) at 37°C. Each value represents the mean optical density (OD) readings from 6 cultures that originated from 2 independent starter cultures. Sub-minimum inhibitory concentration doses used are based on MIC values in Table I.

than 37°C. Interestingly, the MICs for the combination of SMZ + CTC were not much different from the MICs for CTC alone at both 37°C and 41°C, and in many cases they were the same.

Haemophilus somnus 2336 was less susceptible to both CTC and CTC + SMZ than were either of strains 700025 or Hs-91. Febrile temperature rendered strain 700025 more than 4-fold more susceptible to both CTC and CTC + SMZ, whereas only minor temperature effects were seen on the MICs for the 2 clinical isolates. In general, the *H. somnus* strains were more susceptible to both CTC and CTC + SMZ than were *M. haemolytica* strains.

Growth kinetics

Growth curves for the 3 *M. haemolytica* strains reveal typical sigmoidal kinetics with identifiable lag and logarithmic growth phases, as demonstrated by the growth curves at 37°C with CTC (Figure 1). Because of the large variation in growth between antibiotic treatments, we were unable to select a standard OD_{620} range that could be used to calculate generation times. Therefore, we used a manual graphical method for estimating generation times. These estimates, along with the average OD_{620} for each treatment achieved at 5 h post inoculation, reflect the trends in bacterial growth for each strain under the different conditions (Table II).

Growth of the clinical isolates D80 and D152 was severely inhibited in the presence of both antibiotics at 1/2 and 1/4 MICs (in all but 1 case never achieving an OD_{620} of 0.1) (Table II). In fact, growth of strain D152 was severely inhibited at concentrations as low as 1/8 MIC (Figure 1C; Table II). By contrast, nonvirulent isolate 55518 exhibited only moderate to slightly reduced growth at 1/2 and 1/4 MICs (Figure 1A) and, in most cases, there was not much difference between growth without antibiotic compared to growth at 1/8 MIC and 1/16 MIC.

The growth kinetics of the 3 *M. haemolytica* strains were not reduced at 41°C compared to 37°C. Indeed, under some antibiotic conditions, growth kinetics were improved at 41°C compared to 37°C (Table II).

For the *H. somnus* strains, growth curves did not resemble the typical sigmoidal pattern with distinguishable lag and logarithmic growth phases, as demonstrated by the growth curves at 37°C with CTC (Figure 2). In many cases, the bacteria appeared to begin growing without a detectable lag phase. Similar to *M. haemolytica*, generation times could not be calculated from a standard OD_{620} range, so generation times from graphical estimates and mean OD_{620} at 5 h post inoculation are reported (Table III).

Growth of the 3 *H. somnus* strains at 1/2 and 1/4 MICs was limited; in most cases, there was not enough growth to allow estimation

Table II. Estimated generation times and mean optical density (OD_{620}) achieved at 5 h postinoculation of *M. haemolytica* strains cultivated under different antibiotic and temperature conditions

Bacterial strain	CTC 37°C	CTC 41°C	CTC + SMZ 37°C	CTC + SMZ 41°C
M. haemolytica 55518				
No antibiotic	63/0.628[a]	45/0.691[b]	55/0.584	53/0.514[b]
1/16 MIC	65/0.617	48/0.527[b]	60/0.560	55/0.538[b]
1/8 MIC	60/0.560	58/0.491[b]	60/0.515	58/0.489[b]
1/4 MIC	70/0.471	70/0.258[b]	60/0.423	80/0.240[b]
1/2 MIC	98/0.154	115/0.160[b]	60/0.299	138/0.136[b]
MIC	ND[c]/0.063	ND/0.079[b]	ND/0.067	ND/0.064[b]
M. haemolytica D80				
No antibiotic	63/0.495	48/0.618	60/0.475	50/0.581
1/16 MIC	80/0.469	55/0.509	60/0.381	53/0.525
1/8 MIC	65/0.296	60/0.280	75/0.232	58/0.484
1/4 MIC	ND/0.054	ND/0.060	ND/0.054	50/0.258
1/2 MIC	ND/0.058	ND/0.046	ND/0.051	ND/0.049
MIC	ND/0.056	ND/0.046	ND/0.051	ND/0.048
M. haemolytica D152				
No antibiotic	65/0.521	55/0.548	65/0.597	60/0.505
1/16 MIC	63/0.218	75/0.395	60/0.395	58/0.390
1/8 MIC	ND/0.062	ND/0.048	ND/0.085	68/0.134
1/4 MIC	ND/0.053	ND/0.047	ND/0.051	ND/0.048
1/2 MIC	ND/0.056	ND/0.050	ND/0.049	ND/0.047
MIC	ND/0.051	ND/0.052	ND/0.047	ND/0.051

CTC — chlortetracycline; CTC + SMZ — chlortetracycline-sulfamethazine; MIC — minimum inhibitory concentrations

[a] Estimated generation time in minutes/mean OD_{620} achieved at 5 h postinoculation

[b] Reported mean OD_{620} for strain 55518 grown at 41°C were taken at 3.5 h postinoculation; because of the rapid growth of this strain at this temperature, this was the final time point that data was collected

[c] ND (not determined) indicates generation time could not be determined due to poor growth and lack of an identifiable log phase

of generation times. For all 3 strains, growth kinetics at 1/8 and 1/16 MICs for CTC and CTC + SMZ were also inhibited (Figure 2). For 700025 and Hs-91, most cases of growth at 1/8 and 1/16 MICs were markedly inhibited (Figure 2A and 2B).

In the absence of antibiotics, growth kinetics for the *H. somnus* strains were similar at 41°C and 37°C. By contrast, strains 700025 and Hs-91 appeared to have improved growth at 41°C compared to 37°C at some sub-MICs for CTC and CTC + SMZ. However, these strains had much lower MICs for these antibiotics at 41°C compared to 37°C. Therefore, the antibiotic concentrations used for the growth kinetics study at 37°C were higher than those used at 41°C, making it difficult to draw conclusions on the effect temperature had at these sub-MICs.

Capsule production

Comparison of capsule thickness by ANOVA indicated that no significant differences in capsule production occurred for *M. haemolytica* D80 grown in the absence of antibiotic compared to growth in the presence of either CTC or CTC + SMZ at 1/8 MIC and 1/16 MIC at 37°C (Pr > f-value 0.0840). Capsule thickness for D80 grown without antibiotics was 0.63 ± 0.09 μm, 1/16 MIC CTC was 0.56 ± 0.10 μm, 1/8 MIC CTC was 0.65 ± 0.10 μm, 1/16 MIC CTC + SMZ was 0.51 ± 0.12 μm, and 1/8 MIC CTC + SMZ was 0.64 ± 0.11 μm.

Outer membrane protein expression

The SDS-PAGE revealed 4 major OMPs in *M. haemolytica* based on band intensity (Figure 7). The estimated molecular weights of these proteins were 95.9 kDa, 45.0 kDa, 42.2 kDa, and 32.5 kDa. All 4 bands were consistently present in all of the *M. haemolytica* OMP preparations regardless of antibiotic or temperature conditions.

Minor *M. haemolytica* OMPs were also identified that had lower band intensity, including 4 minor bands in the molecular weight range of 19.5 kDa to 27.5 kDa, as well as several minor bands in the 45 kDa to 90 kDa range (Figure 3). Molecular weights of 2 representative OMPs (62.8 kDa and 82.1 kDa) in the 45 kDa to 90 kDa range are shown in Figure 3, and the 4 lower molecular weight minor OMPs were estimated as 30.2 kDa, 28.2 kDa, 26.0 kDa, and 23.6 kDa. There were some variations in the expression of these 4 minor OMPs under different antibiotic and temperature conditions, but no clear trend was evident. There was some difficulty in interpretation due to the low staining intensity of these bands and gel variation.

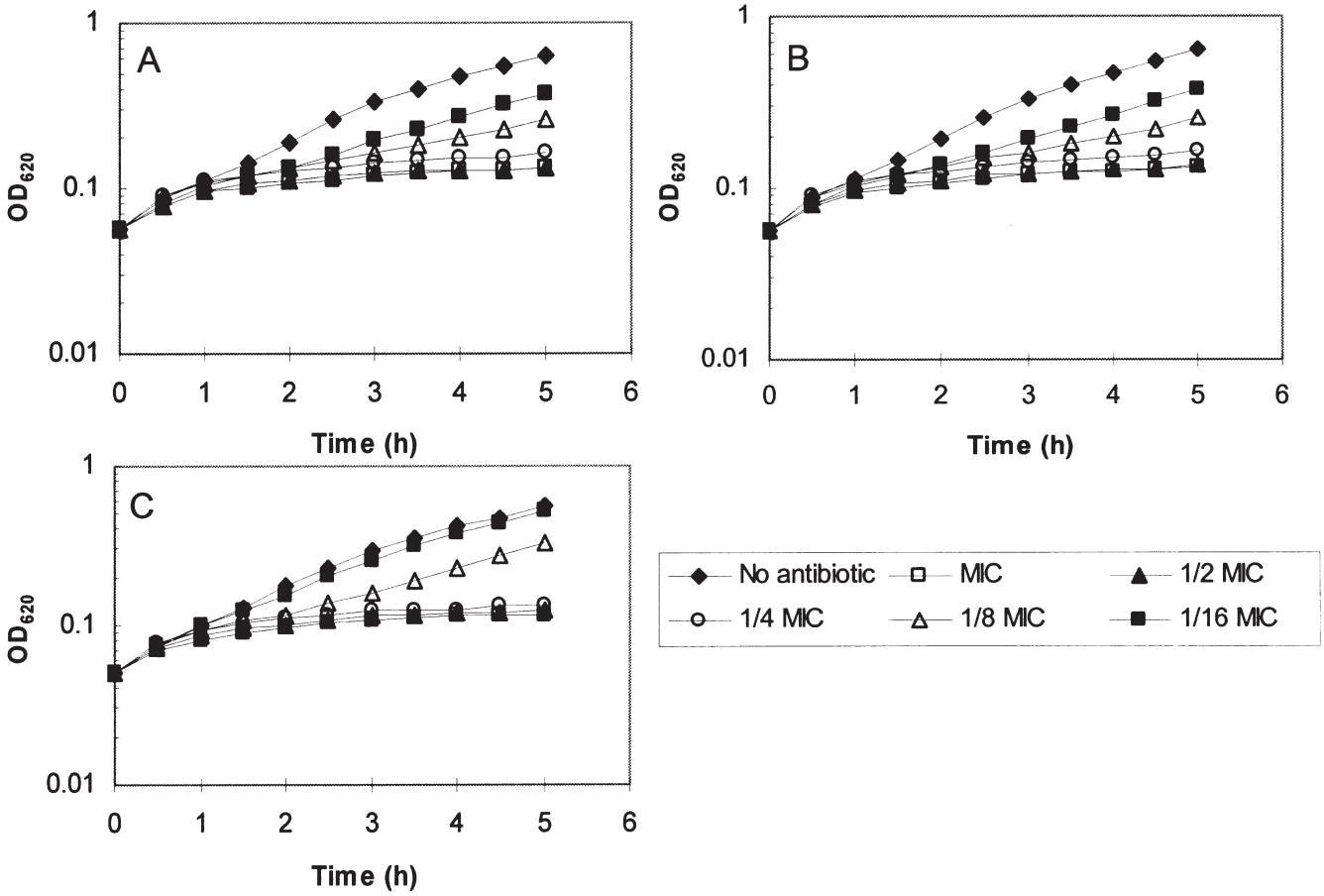

Figure 2. Growth kinetics of *Haemophilus somnus* strain 700025 (A), Hs-91 (B), and 2336 (C) in the presence of sub-minimum inhibitory concentrations (MICs) of chlortetracycline (CTC) at 37°C. Each value represents the mean of three independent determinations. Sub-minimum inhibitory concentration doses used are based on MIC values in Table I.

Therefore, although there was some detectable variation in expression of these 4 OMPs, we could not reliably conclude that sub-MIC antibiotic levels or temperature had an effect on expression of *M. haemolytica* OMPs.

Nine OMP bands were identified in the 3 *H. somnus* isolates (Figure 4). The calculated molecular weights of these proteins were 90.0 kDa, 77.4 kDa, 51.3 kDa, 42.9 kDa, 39.1 kDa, 29.7 kDa, 28.5 kDa, 26.8 kDa, and 14 kDa. These proteins were all consistently expressed by the 3 isolates under all conditions tested.

Discussion

Prophylactic administration of CTC and SMZ at sub-MICs in feed has been demonstrated to significantly reduce the incidence of BRD and increase feed conversion rate in cattle (5). The goal of this study was to determine whether phenotypic changes could be detected in *M. haemolytica* and *H. somnus* as a result of exposure to sub-MICs of CTC and CTC + SMZ at normal and febrile body temperatures, thereby providing a basis for this clinical efficacy.

The MIC results indicated that strain differences had a larger effect on antibiotic sensitivity than temperature or the antibiotic combination used. In particular, the ATCC reference strains tended to be more sensitive than the clinical isolates to CTC and CTC + SMZ, especially at 41°C. This was not surprising and likely reflects adaptation of the ATCC strains to in vitro growth conditions and a decreased ability to adapt to febrile temperature. In addition, our MIC results indicated that *H. somnus* is more sensitive to CTC and CTC + SMZ than *M. haemolytica*.

Studies have indicated that sub-MIC levels of antibiotics reduce the growth rates and increase log phase of bacterial strains (16–19). Our findings were consistent with these earlier reports and demonstrate that the *M. haemolytica* and *H. somnus* clinical isolates had especially limited growth at 1/2, 1/4, and sometimes 1/8 MICs for both CTC and CTC + SMZ. Therefore, although the MIC bioassays demonstrated that the clinical isolates have the ability to grow at higher concentrations of CTC and CTC + SMZ than the ATCC strains, the growth kinetics assays demonstrated that the clinical isolates grow very slowly at the higher antibiotic concentrations (1/2, 1/4, and 1/8 MICs, corresponding to 2 to 16 $\mu g\ mL^{-1}$). This is logical, considering that CTC's mode of action is inhibition of protein synthesis, and sulfonamides inhibit purine and pyrimidine synthesis (through interference with folate metabolism). Inhibition of protein synthesis and purine/pyrimidine synthesis would both be expected to reduce growth rates.

Assuming that similar growth inhibition occurs at equivalent antibiotic concentrations in vivo, our findings suggest a plausible

Table III. Estimated generation times and mean optical density (OD_{620}) achieved at 5 h postinoculation of *Haemophilus somnus* strains cultivated under different antibiotic and temperature conditions

Bacterial strain	CTC 37°C	CTC 41°C	CTC + SMZ 37°C	CTC + SMZ 41°C
H. somnus 700025				
No antibiotic	60/0.654[a]	60/0.469	60/0.668	60/0.574
1/16 MIC	105/0.384	60/0.296	155/0.227	60/0.475
1/8 MIC	180/0.226	90/0.150	ND/0.114	90/0.360
1/4 MIC	ND/0.131	210/0.114	ND/0.096	1000/0.283
1/2 MIC	ND/0.115	ND/0.083	ND/0.074	210/0.166
MIC	ND[b]/0.113	ND/0.068	ND/0.074	ND/0.114
H. somnus Hs-91				
No antibiotic	60/0.634	70/0.352	90/0.565	65/0.561
1/16 MIC	120/0.377	120/0.191	120/0.344	60/0.477
1/8 MIC	180/0.259	ND/0.123	210/0.206	75/0.385
1/4 MIC	390/0.167	ND/0.104	ND/0.136	130/0.168
1/2 MIC	ND/0.135	ND/0.102	ND/0.117	ND/0.120
MIC	ND/0.134	ND/0.097	ND/0.106	ND/0.109
H. somnus 2336				
No antibiotic	75/0.553	60/0.868	85/0.557	60/0.887
1/16 MIC	75/0.519	75/0.784	90/0.483	75/0.741
1/8 MIC	120/0.333	75/0.583	130/0.278	180/0.385
1/4 MIC	ND/0.135	210/0.211	ND/0.150	300/0.191
1/2 MIC	ND/0.124	ND/0.147	ND/0.128	180/0.166
MIC	ND/0.117	ND/0.146	ND/0.128	ND/0.165

CTC — chlortetracycline; CTC + SMZ — chlortetracycline-sulfamethazine; MIC — minimum inhibitory concentrations

[a] Estimated generation time in minutes/mean OD_{620} achieved at 5 h postinoculation

[b] ND (not determined) indicates generation time could not be determined due to poor growth and lack of an identifiable log phase

mechanism for the demonstrated clinical efficacy of sub-MIC administration of these antibiotics. Namely, inhibition of bacterial growth in vivo would slow the progression of infection and presumably allow the host immune system to more readily clear the pathogen.

Earlier reports on the effects of sub-MICs of antibiotics on bacterial growth have not taken into account the effect of growth at febrile temperature. Our finding that growth of the *M. haemolytica* and *H. somnus* isolates was not inhibited (and in some cases improved) at febrile temperature compared to standard in vitro culture temperature was unexpected. The optimal temperature for growth of *M. haemolytica* is reported to be 37°C, with growth occurring between 25°C and 40°C (20). However, our findings are not surprising in light of the fact that febrile response is typical of BRD; in fact, fever is often used as a prognostic indicator. These results indicate that sub-MICs of CTC and CTC + SMZ are effective in inhibiting *M. haemolytica* and *H. somnus* growth at both 37°C and 41°C, thereby suggesting that antibiotic administration is likely to be effective in treatment or prevention of BRD regardless of whether it is given before or after the development of febrile response.

No significant differences were observed in capsule thickness of strain D80 between the different antibiotic treatments. Similarly, tetracycline and trimethoprim/sulphamethoxazole had no effect on capsule production in *P. multocida* (21). However, differences in capsule thickness were observed when *Pasteurella multocida* was exposed to sub-MICs of penicillin G (21). The effect of penicillin on capsule expression in *P. multocida* may be due to its mode of action (inhibition of cell wall synthesis), which contrasts that of the bacteriostatic antibiotics.

The *M. haemolytica* OMPs that were identified in this study are similar to previously reported *M. haemolytica* OMPs. Several investigators have reported a 30 kDa, 30.5 kDa, or 31 kDa OMP from serotype A1 (22–24) that is probably the same as our major 32.5 kDa OMP. Although this protein is readily identified by SDS-PAGE, it could not be clearly identified on Western blots using convalescent calf serum, suggesting it is either not immunogenic or not reactive on Western blots (22). A 44.5 kDa OMP has been reported (23) that is probably the same as our 45.0 kDa major OMP, and a 42 kDa OMP has been reported (24,25) that is probably the same as our 42.2 kDa major OMP. A 100 kDa OMP has been reported (22) that could be the same as our 95.9 kDa major OMP; however, the previously reported 100 kDa OMP is iron regulated, and in our study the 95.9 kDa OMP was well expressed in iron replete BHI. Alternatively, a 92.3 kDa OMP was reported from serotype A2 (24) that could be the same as our 95.9 kDa OMP. One group reported 30.5 kDa, 62.5 kDa, and 82 kDa OMPs (23) that could be the same as some of the minor OMPs we identified (30.2 kDa, 62.8 kDa, and 82.1 kDa).

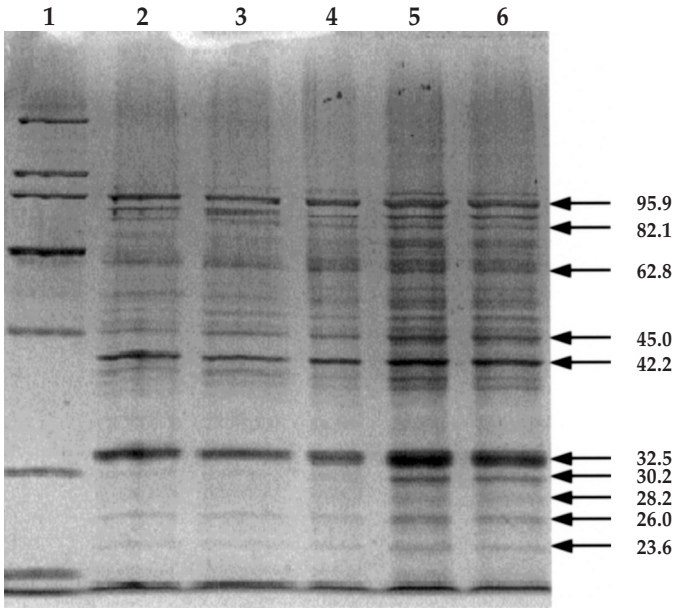

Figure 3. Coomassie stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *M. haemolytica* D80 with sub-minimum inhibitory concentrations (MICs) of chlortetracycline-sulfamethazine (CTC + SMZ) at 37°C and 41°C showing positions of major and minor outer membrane proteins (OMPs) identified in this study. Sub-minimum inhibitory concentration doses used are based on MIC values in Table I. Lane 1, MW marker (Broad Range standard; BioRad Laboratories); lane 2, no antibiotic at 37°C; lane 3, 1/8 MIC at 37°C; lane 4, 1/16 MIC at 37°C; lane 5, no antibiotic at 41°C; lane 6, 1/4 MIC at 41°C.

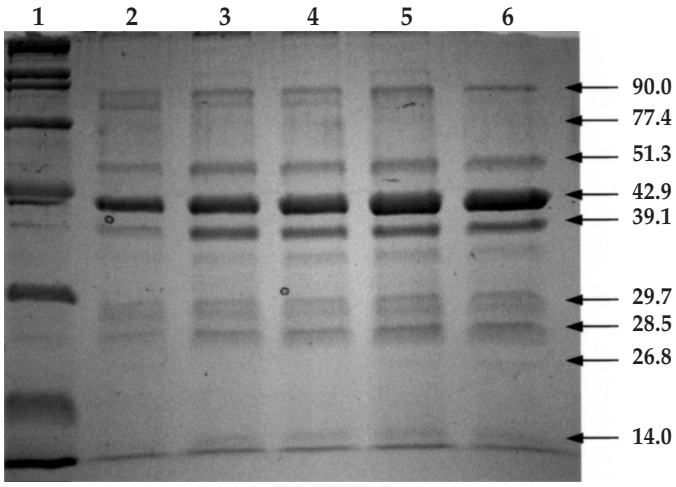

Figure 4. Coomassie stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *Haemophilus somnus* strain 700025 in the presence of sub-minimum inhibitory concentrations (MICs) of chlortetracycline (CTC) at 37°C showing positions of OMPs identified in this study. Sub-minimum inhibitory concentration (MIC) doses used are based on MIC values in Table I. Lane 1, MW marker (Broad Range standard; BioRad Laboratories); lane 2, no antibiotic; lane 3, 1/2 MIC; lane 4, 1/4 MIC; lane 5, 1/8 MIC; lane 6, 1/16 MIC.

Some of the *H. somnus* OMPs identified in the current study can also be putatively identified based on molecular weight comparisons. In a previous study, convalescent calf serum reacted strongly with a 40.0 kDa OMP and a 78 kDa OMP (26), which probably corresponds to the 42.9 kDa OMP and 77.4 kDa OMP identified in the current study. In addition, weaker reactions occurred with 15.0 kDa, 29 kDa, and 31 kDa OMPs, which could correspond with the 14.0 kDa, 28.5 kDa, and 29.7 kDa OMPs identified in the current study. Amino terminal sequencing of the 40.0 kDa OMP indicated that it is a porin (27). Another study identified a 28.0/37.0 kDa heat-modifiable OMP that is reactive with convalescent calf serum (28); this protein could also correspond with the 28.5 OMP detected in the current study. Amino terminal sequencing and immunological cross reactivities indicated that the 28.0/37.0 kDa OMP is similar to OMP-A from *Escherichia coli* K-12 (28), which is a virulence factor that appears to contribute to serum resistance (29).

The OMPs can have important functions that affect the virulence of gram-negative pathogens, such as nutrient uptake (porins or siderophore/heme binding proteins) or adhesion to host cells (30). Variation in expression of *M. haemolytica* OMPs has been reported in response to iron restriction and bacterial growth phase (31) and growth in vivo versus in vitro (22). Temperature and antibiotic effects on OMP expression for either *M. haemolytica* or *H. somnus* have not been reported, although a 54 kDa heat shock inducible protein was reported for *M. haemolytica* (32). In the current study, we did not detect variation in the expression of major OMPs from either *M. haemolytica* or *H. somnus* as a result of growth in the presence of sub-MICs for CTC or CTC + SMZ or as a result of growth at 41°C. Some variation was detected in expression of some of the minor *M. haemolytica* OMPs, but we could not definitively conclude that it occurred as a result of varying environmental conditions.

This study points out the difficulties in using SDS-PAGE to quantify proteins for detecting variation in protein expression. Variation in staining makes comparison of band intensities between gels unreliable, particularly for faint staining bands such as the minor OMP bands from *M. haemolytica*. In addition, accurate protein identifications are difficult with this method due to variation in size estimates that occur as a result of strain variation and differing extraction procedures or gel conditions, making comparison of results between different publications difficult (23). Additional research is needed using more reliable methods such as isotope-coded affinity tags (ICATs) and tandem mass spectrometry (33), which would allow more accurate protein identification and quantification to determine the effects of sub-MIC antibiotic levels and temperature on protein expression in these pathogens.

In this study, it was demonstrated that in vitro sub-MICs of CTC and CTC + SMZ affect the growth kinetics of both *M. haemolytica* and *H. somnus*, offering a plausible mechanism for the demonstrated clinical efficacy of sub-MICs of CTC and CTC + SMZ. It was also shown that growth of *M. haemolytica* and *H. somnus* was not impaired at 41°C compared to 37°C in the presence and absence of antibiotics. No significant effect from antibiotics or temperature was seen on *M. haemolytica* capsule thickness or on OMP expression from either species, but further studies are recommended in order to determine effects on protein expression.

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